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Diisobutyl phthalate has comparable anti-androgenic effects to di-*n*-butyl phthalate in fetal rat testis

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Abstract

Phthalates are widely used as plasticizers in various consumer products and building materials. Some of the phthalates are known to interfere with male reproductive development in rats, and di-*n*-butyl phthalate (DBP), diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) were recently banned for use in toys in the EU mainly due to their reproductive toxicity. Diisobutyl phthalate (DiBP) has similar structural and application properties as DBP, and is being used as a substitute for DBP. However, knowledge on male reproductive effects of DiBP in experimental animals is lacking.

Methods: In the current study, four groups of pregnant Wistar rats were exposed to either 0 mg/kg bw/day or 600 mg/kg bw/day of DiBP from gestation day (GD) 7 to either GD 19 or GD 20/21. Male offspring was examined at GD 19 or GD 20/21 for effects on testicular testosterone production and testicular histopathology. Changes in anogenital distance (AGD) were evaluated as an indication of feminisation of males.

Results: Anogenital distance was statistically significantly reduced at GD 20/21 together with reductions in testicular testosterone production and testicular testosterone content. Histopathological effects (Leydig cell hyperplasia, Sertoli cell vacuolisation, central location of gonocytes and presence of multinuclear gonocytes) known for DBP and DEHP were observed in testes of DiBP-exposed animals at GD 20/21. Additionally, immunohistochemical expression of P450scc and StAR proteins in Leydig cells was reduced by DiBP. At GD 19, these effects on anogenital distance, testosterone levels and histopathology were less prominent.

Conclusion: In this study, GD 20/21 rather than GD 19 appears to be the optimal time for investigating changes in anogenital distance, testosterone levels, and testicular histopathology. DiBP has similar testicular and developmental effects as DBP and DEHP, and although more developmental and especially postnatal studies are needed to clearly identify the reproductive effects of DiBP, this study indicates a reason for concern about the use of DiBP as a substitute for DBP.

Keywords: Phthalic acid ester; Diisobutyl phthalate; Prenatal; Steroidogenesis; Testis; Rats

1. Introduction

The observed increasing incidences of testicular cancer, hypospadia, cryptorchidism and reduced semen quality in recent years have been suggested to be caused by endocrine disrupting environmental compounds with adverse effects on male reproduction (Skakkebaek et al., 2001). Among the chemicals suspected of causing these effects in humans are the phthalates, which are abundantly used in plastics, paints and other materials. Results from a recently published human study indicate associations between maternal phthalate exposure and reproductive development in their infant sons (Swan et al., 2005). Some of the most commonly used phtha-

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lates (dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP) and diisononyl phthalate (DiNP)) are known to induce adverse effects in the reproductive system of male rats after prenatal exposure (Barlow and Foster, 2003; Gray et al., 2000). Some of these effects are likely caused by a reduction in testicular testosterone production during late gestation (Barlow et al., 2003; Borch et al., 2004b; Parks et al., 2000).

Diisobutyl phthalate (DiBP) has not previously been examined in fetal rats, though it is widely used and suspected of having similar reproductive effects as the well-studied DBP. Due to its similarities to DBP, DiBP can be used as a substitute for DBP, which is presently used in PVC, inks, paints, adhesives and cosmetics (European Council for Plasticisers and Intermediates website, www.phthalates.com). Around 100 tons of DiBP is used in Denmark per year mainly as softeners in plastic, rubber, paint and glue, which accounts for 95% of the use. In the US, the monoester metabolite of DiBP, mono-isobutyl phthalate (MiBP), was found in women's urine in levels ranging from 0.7 ng/ml (25th percentile) to 5.1 ng/ml (75th percentile) (Swan et al., 2005). The exposure level of children to DiBP is unknown, but an estimate of the total exposure to DBP in Danish children aged 1–6 years is 400 μg/kg bw/day. This age group is estimated to be the group with the highest exposure level of DBP (Müller et al., 2003).

Since 1999 the six phthalates DEHP, DBP, BBP, DiNP, diisodecyl phthalate (DiDP) and di-*n*-octyl phthalate (DnOP) have been temporarily banned in the EU in the manufacture of toys and childcare articles for children under the age of three because of their carcinogenic, mutagenic and reprotoxic effects. Recently, DBP has along with DEHP and BBP been banned for use in toys irrespective of age categories due to their reproductive toxicity. DiNP, DiDP, and DnOP were concurrently banned in toys that can be put in the mouth by children.

The DiBP metabolite mono-isobutyl phthalate is found in human blood, saliva and urine (Silva et al., 2005). In a recent study, maternal urinary levels of MiBP (among other phthalate monesters) were associated with a short anogenital distance (AGD) in their infant boys (Swan et al., 2005). Anogenital distance is a commonly used measure of demasculinization of rats exposed to anti-androgenic chemicals.

In the current study, AGD, testicular testosterone levels, and testicular histopathology were evaluated in male rat fetuses exposed to DiBP. The effects were examined at gestation day (GD) 19 and GD 20/21 in order to determine the optimal time for detection of effects on fetal testosterone production.

2. Materials and methods

2.1. Test compounds

Diisobutyl phthalate (DiBP), CAS no. 84-69-5, purity 99%, Acros Organics, Geel, Belgium.

2.2. Animals and dosing

Twenty-four time-mated Wistar rats (HanTac:WH, Taconic M&B, Denmark, bodyweight approximately 210 g) were supplied at Day 3 of pregnancy. The day following mating was designated GD 1. The dams were randomized into four groups of eight with similar bodyweight distributions and housed in pairs under standard conditions. Semi-transparent plastic cages with Tapvei aspen bedding were situated in an animal room with controlled environmental conditions (12 h light–dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature 21 ± 2 °C, humidity 50% ± 5%, ventilation 8 air changes per hour). Food (Altromin Standard diet 1324) and tap water were provided ad libitum. Dams were dosed daily by gavage from GD 7 to the day of autopsy with either vehicle (corn oil) or 600 mg/kg bw/day of DiBP. Animals were inspected for general toxicity twice daily. A control group and a dosed group were scheduled for autopsy at GD 19 and a control group and a dosed group for autopsy at GD 21.

Dams were delivered to the animal unit in four blocks mated on consecutive days and autopsied on consecutive days. As two of these blocks were mixed up on arrival, some dams were sacrificed 1 day earlier in pregnancy. Therefore, one fourth of the fetuses autopsied at "GD 21" were only 20 days old, and consequently named GD 20/21. As not all eight mated animals per group were pregnant, $n = 6$ litters per group on GD 19 and $n = 5$ litters in the control group and 6 litters in the DiBP group on GD 20/21.

At the day of autopsy, dams were anesthetized in CO_2/O_2 and decapitated, and fetuses were removed. AGD was measured in all fetuses using a dissecting scope with an ocular reticle. The measurements were performed blinded with respect to treatment group by a skilled technician who has many years of experience in measuring AGD on PND1 pups. Fetuses were decapitated and testes were removed and sampled for histopathology, measurement of testosterone production ex vivo, or measurement of testosterone content.

One or two testes per litter were placed in Bouin's fixative, and one or two testes per litter were placed in neutral buffered formaldehyde for histopathology and immunohistochemistry. One testis per litter was placed in 0.5 ml ice-cold Dulbecco's Modified Eagle Medium/F12 with 15 mM HEPES, 365 mg/l L-glutamine plus 0.1% bovine serum albumin and 0.1 g/l gentamicin. These testes were incubated at 37 °C for 5 h, were then placed on ice, centrifuged at 4000 × *g* for 10 min, and the supernatant was collected and stored at −80 °C until analysis of testosterone content. Testosterone in supernatants was measured without further extraction.

One testis per litter was placed in an empty tube and immediately frozen in liquid nitrogen and stored at −80°C. Steroid